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Inhibition of Glucose-Induced Release of Insulin by Aldose Reductase Inhibitors

(sorbitol pathway/beta cell/glutaric acid/colchicine)

KENNETH H. GABBAY AND WAH JUN TZE

The Cell Biology Laboratory, Endocrine Division, Department of Medicine, Children's Hospital Medical Center; and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **Aldose reductase (alditol: NADP oxidoreductase, EC 1.1.1.21) is the enzyme responsible for the conversion of glucose to its sugar alcohol, sorbitol. In this study, aldose reductase and a closely related enzyme, L-hexonate dehydrogenase (L-gulonate: NADP oxidoreductase, EC 1.1.1.19), were purified from rat pancreas. Glutaric acid, 2,4-dimethyl glutaric acid, 3,3-tetramethylene glutaric acid, and colchicine inhibited both enzymes, albeit with different potencies. These compounds also inhibited both phases of glucose-induced release of insulin by the perfused rat pancreas. The potencies of these inhibitors in depressing the release of insulin correlated with their effectiveness in inhibiting aldose reductase. At higher concentrations of inhibitors, tolbutamide-induced release of insulin was also depressed. The addition of exogenous sorbitol to pancreases treated with glutaric acid restored their ability to respond to glucose and tolbutamide. These findings suggest that the conversion of free intracellular glucose to sorbitol in the beta cell is an essential step in the glucose-induced release mechanism.**

In the sorbitol pathway, two enzymes catalyze the conversion of free glucose to its sugar alcohol, sorbitol, and the further conversion of sorbitol to fructose.

$$\text{D-Glucose} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{Aldose}} \text{D-Sorbitol} + \text{NADP}^+$$

$$\text{D-Sorbitol} + \text{NAD}^+ \xrightarrow[\text{dehydrogenase}]{\text{Sorbitol}} \text{D-Fructose} + \text{NADH} + \text{H}^+$$

Aldose reductase (alditol: NADP oxidoreductase, EC 1.1.1.21) possesses broad specificity for many aldoses, and is characterized by a high K_m for glucose and galactose. In diabetes and galactosemia, the availability of a large pool of free intracellular aldoses causes an increase in activity of the sorbitol pathway. Aldose reductase requires NADPH as a cofactor, which is provided primarily through the activity of the hexosemonophosphate shunt. A nearly stoichiometric relationship between the hexosemonophosphate shunt activity (NADPH production) and sorbitol formation has been demonstrated for lens (1) and nerve (2). Recent studies indicate an important role for the sorbitol pathway in the etiology of diabetic and galactosemic cataract formation (3), neuropathy (4, 5), and tubular nephropathy (6).

The sorbitol pathway is present in the pancreas. Aldose reductase has recently been isolated and purified from beef (7), rabbit (8), and human pancreas (unpublished observation). We have recently localized aldose reductase by immunological methods to the islets of Langerhans (manuscript in preparation). Morrison *et al.* (9) have described the presence of sorbitol and free fructose in isolated rat pancreatic islets in concentrations exceeding those found in plasma or acinar tissues. Whole pancreas also contains NADP-L-hexonate dehydrogenase (L-gulonate: NADP oxidoreductase, EC 1.1.1.19), an enzyme of the glucuronic acid-xylulose shunt with ability to reduce many uronic acids, but with poor affinity for aldoses.

Extensive studies of glucose-induced release of insulin in the islets of Langerhans have thus far failed to indicate the nature of the signal or the metabolic pathway(s) involved. If anything, the constancy of the metabolite concentrations in the islets, in the glycolytic and the hexosemonophosphate pathways during glucose-stimulated release of insulin, is remarkable (10). It is now well established that the beta cell is freely permeable to glucose, and that the intracellular glucose concentration increases very rapidly in response to increased extracellular concentrations (greater than 6-fold in 0.5 min) (10).

Furthermore, a number of investigators have shown that NADPH is necessary for glucose-mediated release of insulin. Electron acceptors (11) and 6-aminonicotinamide (12), agents that are known to interfere with the availability or formation of NADPH, inhibit the glucose-induced release of insulin.

The kinetic characteristics of aldose reductase, the availability of a large pool of free intracellular glucose, and the NADPH requirement for insulin release suggested that the sorbitol pathway may be ideally suited to act as a signal for glucose-induced release of insulin.

The availability of inhibitors of aldose reductase (13) has permitted us to study the role of the sorbitol pathway in the islets of Langerhans. This report describes the identification and partial purification of rat pancreas L-hexonate dehydrogenase and aldose reductase, and the effect of aldose reductase inhibitors on glucose-induced release of insulin by the perfused, isolated rat pancreas. Colchicine, a known inhibitor of glucose-induced release of insulin, is demonstrated to be a potent inhibitor of aldose reductase.

METHODS

Enzyme Isolation. Pancreases were obtained from Sprague-Dawley rats weighing 400–500 g (4- to 6-months old). The tissue was homogenized in a Waring blendor for 2 min with 5 mM Tris·phosphate–5 mM 2-mercaptoethanol buffer (pH 7.4) at a 3:1 (v/w) ratio. The supernatant was collected from homogenates centrifuged at 46,000 × *g* for 30 min. Ammonium sulfate was slowly added to the supernatant to achieve a 30% saturation at 4°. The precipitate was centrifuged and discarded. The resulting supernatant solution was rapidly desalted by passage through a 2.5 × 50-cm column of Sephadex G-25. The desalted solution was pumped onto

Abbreviation: Glt, glutaric acid.

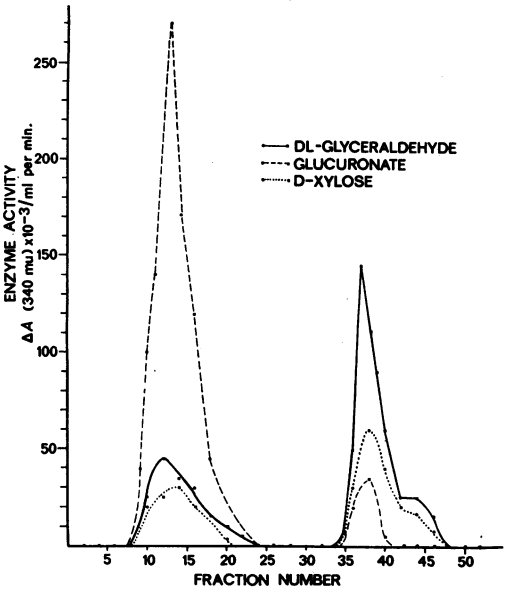

FIG. 1. DEAE-cellulose chromatography of rat pancreas extract (18.5 g of tissue). Column conditions are described in *text*; elution gradient was started at tube 26. The assay mixture (1 ml) contained: phosphate buffer, 67 mM (pH 6.2); 5 mM 2-mercaptoethanol; 0.75 mM NADPH; enzyme solution; and substrate.

a 2.5 × 45 cm column of diethylaminoethyl (DEAE)-cellulose equilibrated with the same Tris·phosphate–2-mercaptoethanol buffer, and the column was eluted with the gradient described by Moore and McGregor (14) at a flow rate of 20 ml/hr. 6-ml fractions were collected and assayed for protein and enzyme activity as described (4). The substrates used were: DL-glyceraldehyde (1 mM), sodium glucuronate (10 mM), and D-xylose (100 mM).

Pancreas Perfusion. Male rats of the CD strain (Charles River Breeding Laboratories) weighing 300–400 g were used for the series of experiments with 3,3-tetramethylene glutaric acid [$(CH_2)_4$Glt] and colchicine. All subsequent perfusions were done with male Sprague–Dawley rats obtained from Sprague–Dawley Co., Madison, Wis. The rats were fasted overnight and anesthetized with Nembutal (50 mg/kg of body weight, intraperitoneally). The pancreases were dissected and perfused as described by Grodsky *et al.* (15). The perfusion fluid was prepared by the addition of salt-poor human albumin (a gift from Dr. George F. Grady, Director, Biologic Laboratories, Massachusetts Department of Public Health) to the solution described by Umbreit *et al.* (16) to achieve a concentration of 4% albumin. A pulsating pump (160 strokes/min) circulated the perfusion fluid in the apparatus, where warming to 37.5° and equilibration with a gas mixture of 95% O_2–5% CO_2 occurred. The resulting pH ranged from 7.26–7.33. Flow rate was maintained at 11–12 ml/min throughout the experiment.

In experiments with aldose reductase inhibitors, glutaric acid (Glt), 2,4-dimethyl glutaric acid [$(CH_3)_2$Glt], and $(CH_2)_4$Glt were neutralized with sodium hydroxide and added to the medium to produce the desired concentration. In both control and inhibitor experiments, the perfusion fluid was recirculated through the pancreas for an exact period of 30 min, during which time the flow rate was adjusted and the preparation was exposed to the particular compound.

At the end of the recirculation period, glucose was infused into the arterial side with a Harvard syringe pump to produce the desired effluent concentration (300 mg of glucose per 100 ml) and samples were taken at 30-sec intervals. Tolbutamide was infused to produce a concentration of about 45 mg per 100 ml of effluent.

Insulin concentrations in the samples were measured in duplicate by the double antibody immunoassay technique (17) with rat insulin as standard (obtained from Dr. Klaus Jorgenson, Novo Terapeutisk Laboratorium). ^{125}I-labeled pork insulin was a generous gift of Cambridge Nuclear Corp., Cambridge, Mass. The highest concentrations used of Glt, $(CH_3)_2$Glt, $(CH_2)_4$Glt, and colchicine did not interfere with the insulin assay.

TABLE 1. *Purification of pancreatic hexonate dehydrogenase and aldose reductase*

	Specific activity (μmol NADPH/ g of protein per hr)	Total activity (μmol NADPH/ hr)	% Recovery
Original extract	36.9	65.1	100
0–30% Ammonium sulfate supernatant	194	125	191
Sephadex G-25 pool	129	67.4	103
DEAE-cellulose			
Hexonate dehydrogenase peak	212	22.4	34.4
Aldose reductase peak	895	38.4	59.6

RESULTS

Enzyme purification

Two main reducing activities were eluted on DEAE-cellulose chromatography of whole pancreas extract (Fig. 1). The first enzyme peak eluted in the buffer wash and contained considerable activity with glucuronate as a substrate. The ratio of the glucuronate-reducing activity to glyceraldehyde-reducing activity was 6:1. The elution pattern and substrate specificities of this enzyme peak indicated that the enzyme

TABLE 2. *Inhibition of pancreatic hexonate dehydrogenase and aldose reductase in vitro*

Inhibitor	Concentration (mM)	% Inhibition: Hexonate dehydrogenase	% Inhibition: Aldose reductase
$(CH_2)_4$Glt	0.05	50	48
Colchicine	0.1	50	21
	0.15	70	56
Glt	1	63	38
$(CH_3)_2$Glt	0.1	50	0
	1	76	6
	2	—	24

Percentage inhibition determined with DL-glyceraldehyde as substrate. Each point is the mean of 2–4 determinations.

is NADP-L-hexonate dehydrogenase (18). A second peak of activity eluting at about a concentration of 60 mM NaCl had a considerably different substrate-activity pattern. The glucuronate-reducing to glyceraldehyde-reducing activity ratio was 1:4. The elution pattern and substrate specificities indicated that the second peak represented aldose reductase. A small shoulder of activity to the main peak of aldose reductase activity was also noted; it differed primarily in having very low glucuronate-reducing activity, as described for bovine kidney aldose reductase (7). A summary of the purification procedures is presented in Table 1; glyceraldehyde activity was used for calculation of the data. We calculated that about 14-fold purification of L-hexonate dehydrogenase, and a 45-fold purification of aldose reductase were achieved. Stimulation of aldose reductase activity by sulfate ions was noted during treatment with ammonium sulfate; the total activity was approximately doubled. Desalting on the Sephadex G-25 column restored the original activity, a phenomenon previously observed with aldose reductases from other sources (19, 20).

Michaelis constants of L-hexonate dehydrogenase and aldose reductase

Pancreatic hexonate dehydrogenase was characterized by its relatively high K_m for D-xylose and D-glucose (110 and 450 mM, respectively). In contrast, aldose reductase had a K_m for D-xylose of 11 mM and for D-glucose of 151 mM. Hexonate dehydrogenase K_ms for DL-glyceraldehyde and D-glucuronate were 1.25 and 0.15 mM, respectively; while the respective K_ms for aldose reductase were 0.18 and 8.3 mM, respectively. These values are similar to those reported in the literature for the two enzymes obtained from other tissues (7, 19, 21), and indeed indicate the proper identification of the two enzyme peaks.

Inhibition of pancreatic L-hexonate dehydrogenase and aldose reductase

Table 2 presents data on the inhibition of hexonate dehydrogenase and aldose reductase by glutaric acid and its analogues $(CH_3)_2$Glt and $(CH_2)_4$Glt. Colchicine, a known inhibitor of insulin release, was also found to inhibit these two enzymes.

It was found that these compounds inhibited both enzymes, but to varying degrees. $(CH_2)_4$Glt was the most potent inhibitor in the series, inhibiting aldose reductase by 48% at a concentration of 0.05 mM. Colchicine was more potent

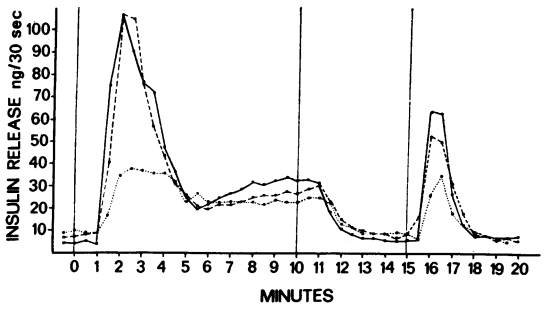

FIG. 2. Effect of 7.5 mM Glt and 7.5 mM $(CH_3)_2$Glt on insulin release. Glucose was infused from 0 to 10 min, shutoff from 10 to 15 min, followed by infusion of tolbutamide from 15 to 20 min. In this and all subsequent experiments, the perfusate was substrate-free until infusion was started at 0 min. Control ——, N = 4; 7.5 mM $(CH_3)_2$Glt – – –, N = 4; 7.5 mM Glt · · ·, N = 3.

TABLE 3. *First phase insulin release*

Inhibitor (mM)	N	Insulin output during first 5 min of glucose infusion ng ± SEM	
Control	5	750	101
$(CH_2)_4$Glt			
10	3	338	103 $P < 0.05$
15	3	172	52.8 $P < 0.001$
20	4	221	64.7 $P < 0.005$
Colchicine			
1	4	400	89.9 $P < 0.05$
2	3	173	3.0 $P < 0.001$
3	3	129	22.2 $P < 0.001$
Control	4	545	70
Glt			
5	3	521	151 NS*
7.5	3	284	23.4 $P < 0.02$
10	4	98	20.6 $P < 0.001$
$(CH_3)_2$Glt			
5	3	508	49.1 NS
7.5	4	509	78.6 NS
10	3	152	57.8 $P < 0.005$

* NS, Nonsignificant.

in inhibiting hexonate dehydrogenase than aldose reductase, as were Glt and $(CH_3)_2$Glt. $(CH_3)_2$Glt, a key compound in this series, inhibited hexonate dehydrogenase by an average of 50% at a concentration of 0.1 mM, but did not affect aldose reductase. This dichotomy persisted with virtually no inhibitory effect on aldose reductase until a concentration of $(CH_3)_2$Glt of 2 mM was used, at which point an average 24% inhibition was observed. Since the location of hexonate dehydrogenase in the pancreas is not known, and since $(CH_2)_4$Glt, Glt, and colchicine inhibited both enzymes, the dichotomy of the inhibitory potency of $(CH_3)_2$-Glt on hexonate dehydrogenase and aldose reductase was especially useful in determination of the specificity of action of these compounds in the perfused rat pancreas.

Effect of $(CH_2)_4$Glt, Glt, and $(CH_3)_2$Glt on insulin release (Figs. 2 and 3, and Table 3)

Fig. 2 shows that first phase release of insulin in response to glucose stimulation began 90 sec after the start of the glucose pulse, reached a peak at 120 sec, and declined to the lowest value by 5 min; this was followed by a gradually rising rate of insulin secretion (second phase) up to 10 min of glucose stimulation. Glucose pulse shutoff led to decreasing insulin output beginning at 150 sec and reaching prestimulatory insulin levels 3 min after shutoff. The subsequent response to tolbutamide infusion consistently reached a peak at 60 sec with subsequent decline to baseline levels at 3 min.

Pancreases treated with increasing concentrations of $(CH_2)_4$Glt showed progressive inhibition of glucose-induced insulin response (pattern not shown). At 10 mM of $(CH_2)_4$-Glt, insulin response was reduced by 50% (Table 3). At 15 and 20 mM $(CH_2)_4$Glt, the insulin response was markedly attenuated, although a small delayed and broadened response was still seen. There were no differences in the patterns or amounts of insulin secretion between the control pancreases

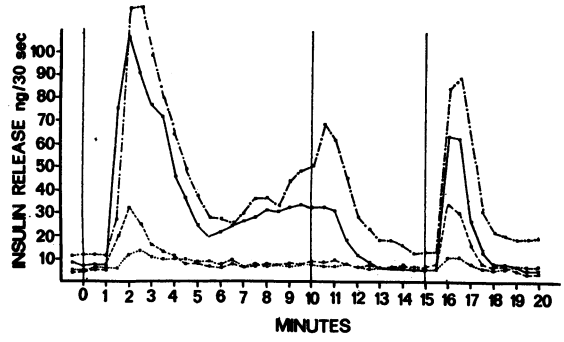

FIG. 3. See Fig. 2. Control ——, N = 4; 10 mM $(CH_3)_2$Glt – – –, N = 3; 10 mM Glt · · ·, N = 4; 10 mM Glt + sorbitol 300 mg/100 ml —·—·, N = 3.

and those treated with 5 mM Glt or 5 mM $(CH_3)_2$Glt (Table 3).

Fig. 2 also shows the results obtained by use of 7.5 mM of each of Glt and $(CH_3)_2$Glt. Insulin release by pancreatic preparations exposed to 7.5 mM $(CH_3)_2$Glt was normal with no diminution or alteration in pattern. However, 7.5 mM Glt [a more potent inhibitor of rat aldose reductase than $(CH_3)_2$Glt] showed a significant inhibition of first phase insulin release and a decrease (flattening of the curve) of second phase insulin secretion. The tolbutamide-induced insulin release by the 7.5 mM Glt-treated preparations, though diminished, was not significantly different from the insulin release by control preparations.

Fig. 3 shows the effects of 10 mM Glt and 10 mM $(CH_3)_2$Glt on insulin secretion. At these concentrations, an inhibitory effect on rates of first phase and second phase insulin release was noted with both compounds, although Glt showed greater inhibition. In both instances, second phase insulin-release response was practically absent. Tolbutamide-induced insulin release was significantly decreased (41%, $P < 0.05$) in pancreases treated with 10 mM $(CH_3)_2$Glt, and even further depressed with 10 mM Glt (65%, $P < 0.001$).

The addition of sorbitol (300 mg/100 ml) during the 30-min recirculation period to pancreases treated with 10 mM Glt restored the ability of the pancreases to respond to glucose stimulation (Fig. 3). Both phases of insulin release, as well as subsequent tolbutamide response, were restored to normal. Sorbitol by itself did not stimulate the release of insulin.

Effect of colchicine on release of insulin (Fig. 4 and Table 3)

Fig. 4 demonstrates the effect on insulin release of colchicine perfusion for 30 min before stimulation with glucose. The data show that increasing concentrations of colchicine progressively inhibited both the first phase and second phase of insulin secretion as compared to the control perfusions. Additionally, at concentrations of 2 and 3 mM colchicine, there was a delay in reaching the peak of glucose-induced insulin secretory rates to 3.5 min as opposed to the normal 2 min. Second phase insulin release was practically eliminated by treatment with 3 mM colchicine. Tolbutamide-induced insulin release was not depressed.

DISCUSSION

Glucose-induced insulin release is a biphasic process, while tolbutamide induces only a single insulin secretory phase. The biphasic insulin response to glucose was described in studies with the perfused rat pancreas (15), in dog (22), and man (23). Our data show that the peak insulin response to tolbutamide consistently occurs a full 60 sec earlier than the peak response to glucose, suggesting that tolbutamide exerts its action at a later step in the stimulatory pathway than glucose itself. Further experiments in this laboratory (in preparation) indicate that the superimposition of a glucose infusion (300 mg/100 ml) on a tolbutamide infusion in progress (45 mg/ml) fails to induce additional insulin release. The opposite is also true; the superimposition of a tolbutamide pulse on a maximal glucose infusion also fails to produce significant additional insulin release. These findings support the concept that both glucose and tolbutamide share a final common step in the pathway for insulin release.

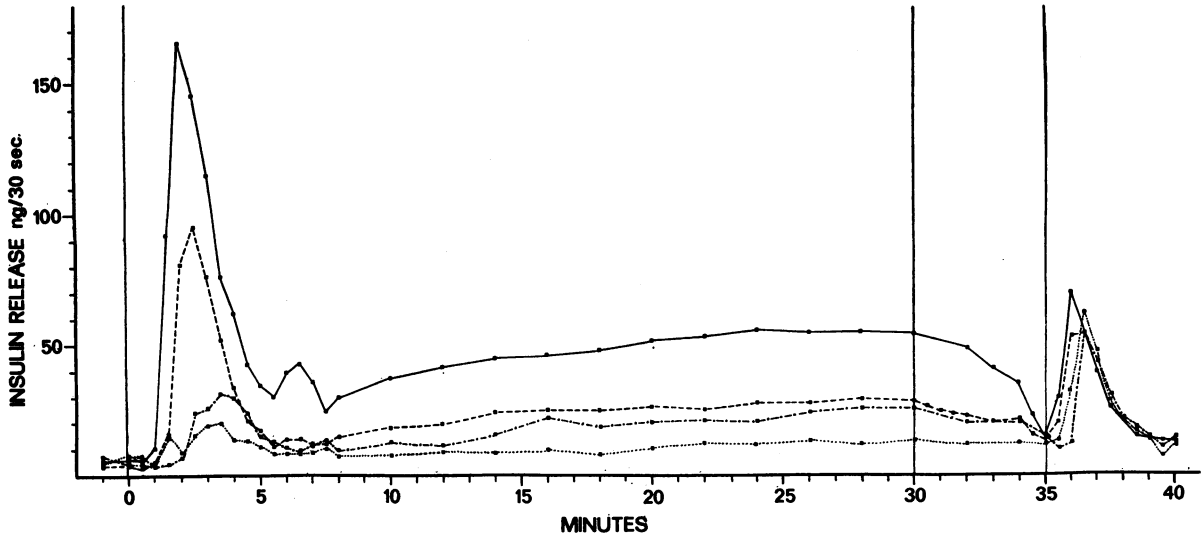

FIG. 4. Effect of increasing concentrations of colchicine on insulin release. Glucose was infused from 0 to 30 min, shutoff from 30 to 35 min, and tolbutamide was infused from 35 to 40 min. Control ——, N = 5; Colchicine: 1 mM – – –, N = 4; 2 mM —·—·, N = 3; 3 mM · · ·, N = 3.

The nature of the sequence of events originated by glucose in the pathway for insulin release is unknown. Clearly, glucose has a dual role, as a signal and as a source of energy for the release mechanism. Matschinsky *et al.* (10, 24) demonstrated that intracellular free glucose in rat islets rises rapidly in response to ambient glucose concentrations, and that glucose phosphorylation is the rate-limiting step in glucose utilization. The data presented in this study suggest that the conversion of intracellular free glucose to sorbitol by aldose reductase is the initial step (or signal) in insulin release. Aldose reductase, by virtue of its high K_m for glucose (150 mM), is well suited to linearly detect changing concentrations of glucose over the physiological stimulatory range. The dual requirement of glucose-induced insulin release (11, 12) and aldose reductase for NADPH additionally supports this concept. Since NADPH in islets is provided mainly through the metabolism of glucose-6-phosphate via the hexosemonophosphate shunt, interference with glucose phosphorylation, not surprisingly, also leads to inhibition of glucose-induced insulin release. The findings of Renold and coworkers (25) that inhibition of glucose phosphorylation by mannoheptulose or 2-deoxyglucose paradoxically potentiates tolbutamide-induced insulin release, despite blocking glucose-induced release, may be explained by shunting of glucose into sorbitol formation. Our finding that higher concentrations of Glt and $(CH_3)_2$Glt depress tolbutamide-induced release as well is consistent with the idea that sorbitol is required to "prime" or activate an element in the insulin release mechanism of the beta cell.

This view is supported by our finding that simultaneous exposure of the pancreas to both Glt (10 mM) and sorbitol (300 mg/100 ml) restored insulin responses to normal (Fig. 3). Preliminary experiments indicate that galactitol is not as effective as sorbitol in restoring insulin responses. One possible mechanism for such an effect of sorbitol is that it directly interacts with and activates a protein in the beta cell (e.g., microfilaments). Sorbitol has been shown to specifically and stoichiometrically induce an irreversible conformational change in lens alpha crystallin (26).

The above interpretation is dependent on the specificities of action of the aldose reductase inhibitors used in this study. With the exception of $(CH_2)_4$Glt, there is a good correlation between *in vitro* inhibitory potency on aldose reductase by Glt, $(CH_3)_2$Glt, and colchicine, and their ability to inhibit the release of insulin. The effectiveness of these inhibitors obviously depends on their penetration into the islets during the recirculation period (30 min) before the infusion of glucose. Increasing the period of exposure to 90 min with 1 mM colchicine caused complete inhibition of both phases of insulin release. The poor penetrability of $(CH_2)_4$Glt into tissues is supported by data obtained in lenses incubated in media with high concentrations of galactose that showed that the presence of the tetramethylene ring structure in $(CH_2)_4$Glt limits its ability to penetrate cell membranes (20). Glt and $(CH_3)_2$Glt do not possess this ring structure and presumably would penetrate the cell membrane more freely. 10 mM of $(CH_2)_4$Glt has been shown to block sugar alcohol formation in lenses (27) and sciatic nerves (2) incubated in media with high concentrations of glucose. No interference by $(CH_2)_4$Glt with normal glucose metabolism nor any effect on a number of glycolytic enzymes could be demonstrated (13). Furthermore, the probable specificity of colchicine in inhibiting aldose reductase is indicated by the finding that the incubation of lenses in media with high concentration of galactose containing 1 mM colchicine induces the same degree of inhibition of galactitol formation as that produced by 10 mM $(CH_2)_4$Glt (Gabbay and Kinoshita, unpublished observations). At the present time it is not clear whether the effect of colchicine on release of insulin is due to inhibition of aldose reductase and/or due to its effect on microtubules. It is possible that microtubular function may depend on aldose reductase activity.

These findings suggest that the sorbitol pathway is an important and basic mechanism in the islet cell. Furthermore, the previous demonstration of a role for the pathway in the formation of a number of diabetic and galactosemic tissue manifestations and its presence in a wide variety of tissues suggests a general and basic importance for this pathway in normal cellular metabolism.

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